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13. ABSTRACT (Maximum 200 Words)

Breast cancer is the second deadliest cancer in US women, with estimated 182,800 new cases yearly. PTEN has been characterized as a tumor suppressor gene and found deleted or mutated in many human tumors, including breast, and functions to negatively regulate cell growth, migration, etc, through down-regulation of downstream mediators, such as Akt, etc. Germline PTEN mutations are associated with Cowden syndrome, characterized by increased risk of developing breast cancer. PTEN expression has a positive ER and PR status in primary breast cancers. Whereas, approximately 65% of tumors tested are positive for ER and PR and 75%-80% positive for AR. Androgen, through AR, inhibits mammary carcinoma growth in animal models and is used clinically to influence breast cancer progression. AR germline mutations can cause partial androgen insensitivity. Combined with BRCA1 germline mutations associated with earlier age onset breast cancer, AR functional changes are implicated in breast cancer, suggesting PTEN and AR play roles in breast cancer progression. However, detailed correlations remain unknown. We propose the PTEN pathway, by AR interaction, results in AR-mediated cell growth modulation, and provides a new molecular mechanism of PTEN-mediated AR suppression signaling pathways. Our studies may provide new gene therapies/drug designs for breast cancer patients.

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Table of Contents

Introduction

PTEN has been characterized as a tumor suppressor gene and commonly found to be deleted or mutated in a variety of human tumors, including breast cancer. PTEN functions with a dual-specificity protein and lipid phosphatase and negatively regulates cell growth, migration, invasion, and focal adhesions through down-regulation of downstream mediators, such as Akt, focal adhesion kinase, and Shr. Consistent with its tumor suppressor role, overexpression of PTEN in breast cancer cell lines may result in the suppression of the cell growth. Additionally, germline mutations in PTEN are associated with Cowden syndrome (CS), a rare autosomal disease characterized by an increased risk of developing breast cancer. On the other hand, androgen, acting through the androgen receptor (AR), has been suggested to inhibit mammary carcinoma growth in animal models and has been used clinically as an adjuvant therapeutic agent to influence breast cancer progression. In addition, the germline mutation of the *AR* gene has been shown to cause partial androgen insensitivity together with familial male breast cancer, and the longer CAG repeat (>28) within the *AR* gene (causing lower AR activity) combined with BRCA1 germline mutations is associated with an earlier age of onset of breast cancer development, suggesting that loss or reduction of AR function is implicated in the development of breast cancer. AR has been suggested to be importantly involved in breast cancer initiation. It also has been found that approximately 75%-80% of primary breast tumor specimens tested are positive for AR. Because PTEN expression is correlated with a positive ER and PR status in primary breast tumors and frequently reduced in advanced stage of breast cancer, it is conceivable to propose that PTEN counteracts AR in the mammary tissue to increase the susceptibility to transform to malignancy in the early stage of breast cancer development. Therefore, it will be interesting to study the relationship between PTEN and AR in breast cancer and to determine if PTEN may modulate cell growth through AR.

This suggests that both PTEN and AR play important roles in the progression of breast cancer. However, the correlation between PTEN and AR in breast cancer remains unknown. Therefore, this study provides not only a new molecular mechanism of PTEN-mediated AR suppression signaling pathways, which implicates the suppression of cell growth in breast cancer, but also has therapeutic implications, such as gene therapy or drug design, for breast cancer patients in the future. Our objective is identifying a new PTEN pathway by direct interaction with AR that may result in the suppression of AR-mediated cell growth. The detailed mechanism of how PTEN can repress AR transactivation will also be dissected. The scope of our work includes the following Aims.

Aim 1: To determine that PTEN suppresses AR transactivation and causes growth suppression in MCF-7 cells,

Aim 2: To demonstrate that the direct interaction of PTEN and AR represents a new signaling pathway and to determine whether PTEN directly dephosphorylates AR as a PTEN phosphatase substrate,

Aim 3: To determine the molecular mechanisms of how PTEN can repress the AR transactivation.

These aims will be accomplished by a) determining the suppressive effect of PTEN and PTEN-C124S on AR transactivation and the growth of MCF-7 cells, b) determining the interaction domains between PTEN and AR, c) proving the *in vivo* interaction of PTEN and AR in co-immunoprecipitation assay in MCF-7 cells, d) performing the co-localization of PTEN and AR in MCF-7 cells to understand in which compartment PTEN functions on AR, d) establishing the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR, and e) determining that PTEN reduces AR protein expression.

BODY Statement of Work

Task 1(1 year): Determine the suppressive effect of PTEN and PTEN-C124S on AR transactivation and the growth of MCF-7 cells.

a. Determine endogenous AR-targeted p21 mRNA expression in MCF-7 cells transfected with PTEN and PTEN-C124S
b. Construct plasmids such as pTet-on-PTEN and pTet-on-PTEN-C124S
c. Establish MCF-7 cells stably transfected with vector, PTEN, and PTEN-C124S by neomycin selection
d. Determine MTT growth assay and Northern Blotting detecting p21 mRNA expression

Task 2 (1 year): Determine the interaction domains between PTEN and AR

a. Construct series of PTEN cDNA fragments into GST expression vectors
b. Construct series of AR cDNA fragments into pET-28c expression vectors
c. Determine the AR-binding sites of PTEN and PTEN-binding sites of AR in GST pull-down assay

Task 3 (0.5 year): Prove the *in vivo* interaction of PTEN and AR in co-immunoprecipitation assay in MCF-7 cells.

Task 4 (0.5 year): Perform the co-localization of PTEN and AR in MCF-7 cells to understand in which compartment PTEN functions on AR.

Task 5 (0.5 year): Establish the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR.

Task 6 (0.5 year): Establish the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR.

a. Determine whether PTEN influences AR protein expression by enhancing AR degradation in pulse-chase assay.
b. Determine whether the AR instability is enhanced by dephosphorylation of AR by PTEN or by caspase- or ubiquitinase-mediated protein degradation.
c. Determine whether PTEN also influences AR protein expression at transcriptional or translational levels in Nuclear Run-on assay.

Progress reports

A. From 1st years report:

Our studies have involved the MCF-7 breast cancer cell line and results are best summarized in the two figures presented below.

In Figure 1, we demonstrate that AR and PTEN are endogenously expressed in MCF7 cells. And we found that these two proteins can interact with each other in this cell line by co-immunoprecipitation.

As shown in Figure 2, using GST pull-down assay, we found that PTEN and AR are associated directly via the PTP domain of PTEN and AR DNA binding domain.

These studies were reported in a poster format at the Era of Hope Symposium in Orlando, Florida, September 25-28, 2002 and will be submitted along with other results to a peer reviewed Journal in the near future.

Figure 1.

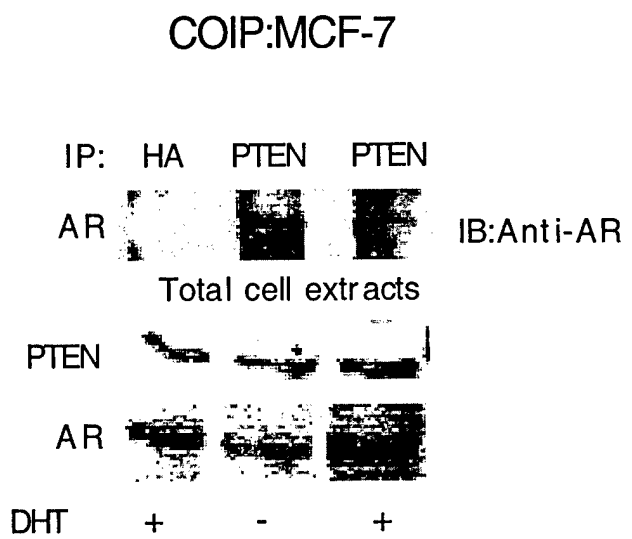
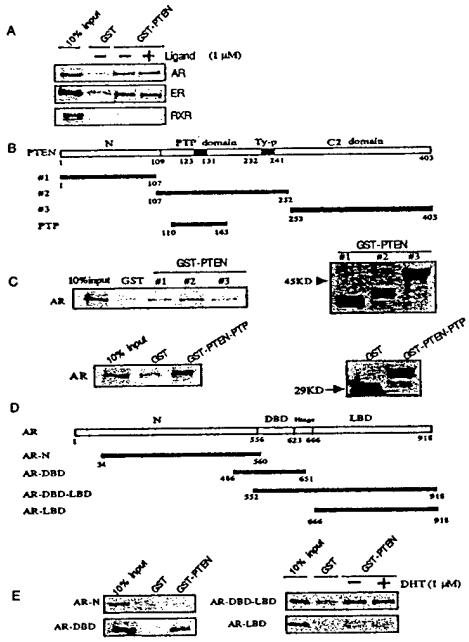


Figure 2.



Legend Figure 1. The MCF-7 cells were cultured in RPMI with 10% Charcoal-dextran treated fetal bovine serum media for 16 hours and treated with ethanol or 10 nM DHT for another 16 hours. Then, cells were harvested and subjected to co-immunoprecipitation with anti-PTEN and anti-HA (control) antibodies, followed by Western Blotting with AR and PTEN antibodies. Results shown are representative of several individual experiments.

Figure 2. GST or GST-PTEN incubation with the [^{35}S]-labeled AR, ER, or RXR for 2 hours in the presence or absence of the ligand. The bound proteins were analyzed by SDS-PAGE, followed by autoradiography. B. Representation of PTEN deleted mutants. PTP domain, protein tyrosine phoshates domain; ty-p, tyrosine phosphorylation domain. C. [^{35}S]-labeled AR was incubated with different PTEN deleted mutants. The nearly equivalent aliquots of PTEN deleted mutants used are shown in the right panel. D. Representation of AR deleted mutants. E. GST or GST-PTEN was incubated with different AR deleted mutants. The SDS-PAGE data represents results from several individual experiments.

B. Second report Progress towards Task 1, Task 2 and Task 3: Previous results showed that PTEN can suppress AR transcriptional activity, and PTEN endogenously interacts with AR in MCF-7 breast cancer cell lines. Because MCF-7 cells contain both PTEN and AR, we can not directly use this cell line to demonstrate the changes of AR nuclear translocation in the absence or presence of PTEN. Therefore, we studied a common used cell line COS-1, which does not contain PTEN and AR. (see Figure 3). We can look at how PTEN influences AR simply by transient transfection of AR with or without PTEN. We found that PTEN could reduce AR protein levels through increasing AR protein degradation rate (Figure 3) in COS-1 cells.

Also, we could apply AR-D, an interaction inhibitor, to see if it could block the PTEN effect on AR. Once we find the positive results using COS-1 cells, we will study them in MCF-7 cells using PTEN siRNA to suppress endogenous PTEN to see if it affects AR translocation. As shown in Figure 3, we indeed saw that PTEN inhibits AR nuclear translocation and AR-D can prevent this inhibition. Next, to test the suppression efficiency of several PTEN siRNA vectors that we have constructed on endogenous PTEN expression, we applied them in 293T cells because this cell line contains endogenous PTEN and most importantly, the transient transfection efficiency is very high (more than 50%) using SuperFect (Qiagen). We demonstrated that overexpression of PTEN promotes AR degradation and suppresses AR activity. To avoid the obtained observations resulting from overexpression, we used small interfering RNA (siRNA) to block endogenous PTEN and examined whether the AR protein levels and transcriptional activity would be affected by downregulating of PTEN. As shown in Figure 4A, transient transfection of PTEN siRNA reduced endogenous PTEN protein levels up to 50-60%, which correlated with the transfection efficiency (around 50%) in 293T

cells. As expected, reduction of PTEN expression enhanced AR protein expression in the presence and absence of androgen (Figure 4A). Likewise, PTEN siRNA dose-dependently enhanced AR transactional activity (Figure 4B). These results suggest that endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity.

Progress towards Task 4. To further investigate whether PTEN can co-localize with AR and then influence AR nuclear translocation we assessed the immunocytofluorescence staining in COS-1 cells (Figure 5A-D). Again, we could not directly use MCF-7 to show changes of AR nuclear translocation in absence or presence of PTEN..

Progress towards Task 5: None

Progress towards Task 6. 6a only. To determine whether PTEN influences AR protein expression by enhancing AR degradation in pulse-chase assay in COS-1 cells. Again, we could not directly use this MCF-7 to demonstrate the changes of AR nuclear translocation in the absence or presence of PTEN. As shown in Figure 4B, PTEN clearly reduced the half-life of newly synthesized [35S]-AR 4 to 5-fold and accelerated AR degradation. Consistently, inhibiting endogenous PTEN expression by PTEN siRNA can increase AR protein level (Figure 4A).

Figure 3

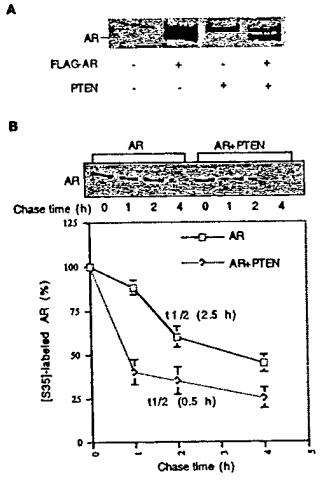
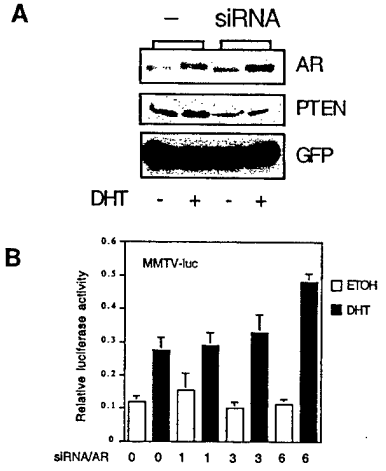


Figure 4



Legend Figure 3. (A) COS-1 cells were transfected with AR flag epitope in front of the AR sequence, along with pCDNA3 or PTEN in 10% CDS media for 16 h and harvested for Western blot analysis. (B) COS-1 cells were transfected with Arand pCDNA3 or PTEN in 10% CDS media for 16 h, pulsed with [^{3}5S]-methionine for 45 min in the presence of 10 nM DHT, harvested at different chase times, extracts immunoprecipitated with AR antibody, subjected to SDS-PAGE, followed by autoradiography. A is a blot representing several experiments and in B is the mean ± SD of at least three experiments.

Legend Figure 4. Endogenous PTEN negatively regulates AR protein stability and transcriptional activity. (A) 293T cells were transfected with PTEN siRNA along with AR for 24 h, followed by ethanol or 10 nM DHT treatment for another 24 h, and harvested for Western blot analyses. (B) 293T cells were transfected with various amounts of PTEN siRNA with AR and MMTV-luc for 24 h, followed by ethanol or 10 nM DHT for 24 h, and harvested for luciferase assays. A is typical of several experiments and B is the mean ± SD of at least three experiments.

PTEN co-localizes with AR *in vivo* and prevents AR nuclear translocation.

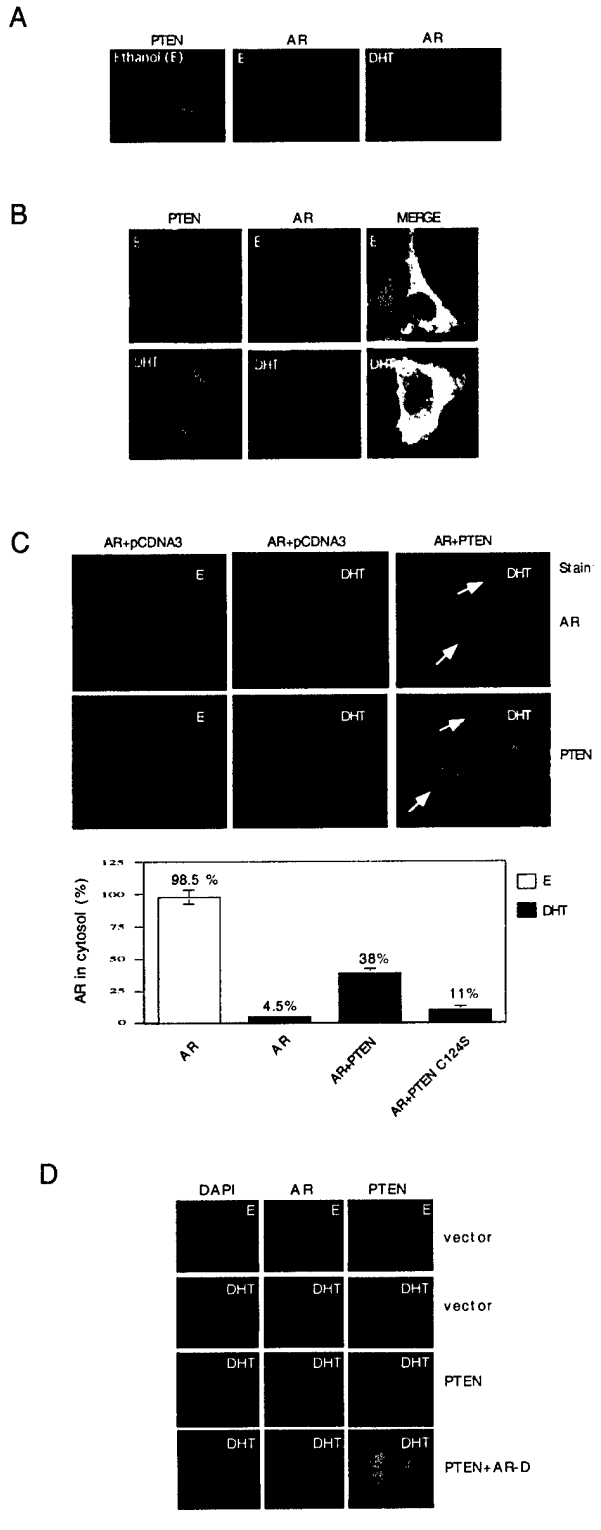


Figure 5 (A - D)

(A,B) The COS-1 cells were transfected with AR or PTEN in 10% CDS media for 16 h and treated with ethanol or 10 nM DHT for another 16 h. The cells were fixed and stained with AR and PTEN antibodies, followed by examination with confocal microscopy. The green and red colors represent PTEN and AR staining, respectively, and the yellow color represents PTEN and AR colocalization. (C) The arrows indicate PTEN positive cells, which show AR located in the cytosol. At least 150 cells were scored for each sample, and data are means ± s.d. from three independent experiments. (D) The COS-1 cells were transfected with AR in combination with plasmids, as indicated for 16 h, followed by 10 nM DHT treatment for another 16 h. The cells were fixed for immunostaining.

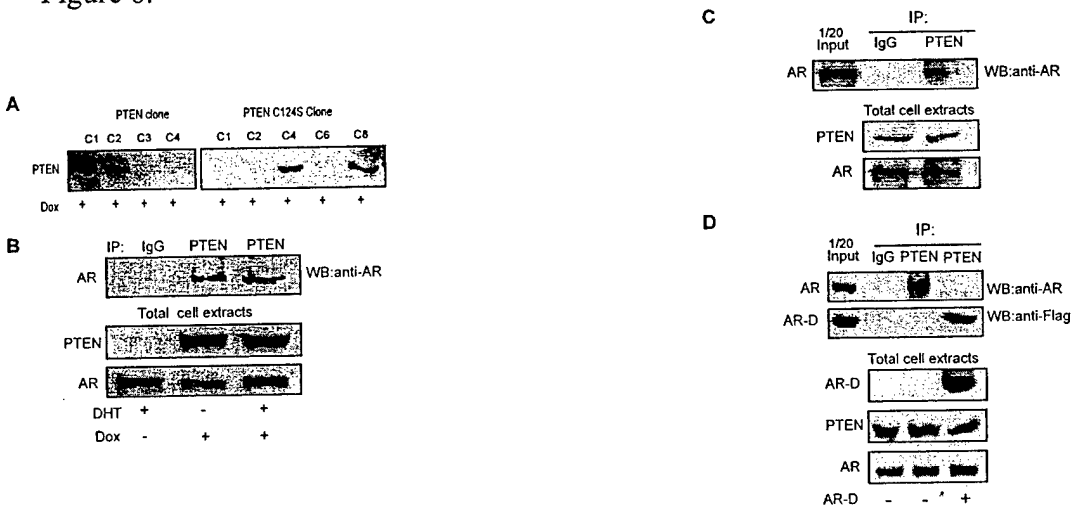
The interaction between PTEN and AR was also analyzed by the subcellular co-localization study, using fluorescence immunostaining. As shown in Fig.5 A, the fluorescent FITC-stained PTEN was mainly located in the cytosol, but small amounts of PTEN were also found in the nucleus. Similar to the FITC-stained PTEN, Texas-RED-stained AR was also mainly located in the cytosol in the absence of androgen, but androgen treatment caused AR nuclear translocation (Fig.5 A). Fig. 4B further demonstrates that PTEN could co-localize with AR in the presence or absence of androgen. Interestingly, we found that PTEN significantly blocked AR nuclear translocation in response to androgen and increased the AR retention (from 4% to 38%) in the cytosol. In contrast, PTEN C124S only showed a slight inhibition of AR nuclear translocation (Fig.5 C).To further prove that PTEN suppression of AR function may go through direct PTEN-AR interaction, we utilized AR-D, which can interact with PTEN and disrupted the interaction between AR and PTEN in the CWR22R cells (Fig. 5D), for functional studies. Our results further showed that AR-D could dramatically reduce PTEN-mediated inhibition of AR nuclear translocation.

Final Year's Progress:

During this final year of the grant we continued to have difficulties using breast cells, but obtained further information regarding the PTEN mechanisms. We include this data here fully understanding it could only be used as preliminary information towards future studies on breast cancer cell lines.

To further confirm the physiological interaction between AR and PTEN by co-immunoprecipitation, we established PTEN-stable LNCaP cells, using the Dox-inducible system. Dox treatment induced expression of PTEN or PTEN C124S in several clones (PTEN-C1, PTEN-C2, PTEN C124S-C4, and PTEN C124S-C8, Figure 6A). PTEN could be co-immunoprecipitated with AR, when we used cell lysates from PTEN-C1 cells (Figure 6B). To rule out the possibility that PTEN antibody may cross-react with AR, we demonstrated that PTEN antibody did not pull-down AR, using parental LNCaP cells, which express AR but not PTEN (data not shown). To further prove that endogenous PTEN can interact with the endogenous AR in the prostate cancer cell line, we applied the CWR22R cell line (1, 2), which endogenously expresses both AR and PTEN (Figure 6C), for co-immunoprecipitation with PTEN antibody. The results showed that AR could be detected in the PTEN-immunoprecipitated complex (Figure 6C). To further determine whether the DBD plus the hinge region of AR (aa, 486-651, AR-D) can interact with PTEN, we transfected AR-D into CWR22R cells for co-immunoprecipitation with PTEN antibody. We found AR-D could be found in PTEN-immunoprecipitated complex (Figure 6D). Interestingly, AR-D could also prevent endogenous PTEN from binding to AR in CWR22R cells (Figure 6D). These results suggest that AR can physiologically interact with PTEN through the AR-D region in prostate cancer cells.

Figure 6.



Legend Figure 6. PTEN Interacts with AR *in vivo*

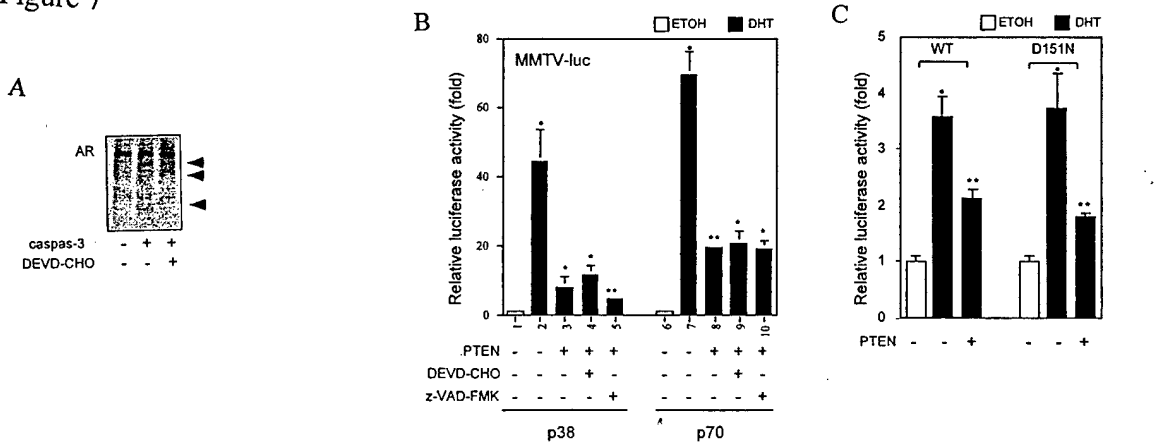
A, The establishment of stable PTEN and PTEN C124S clones in LNCaP cells using doxycycline (Dox)-inducible system. The cells were treated with 4 μg/ml Dox for 24 h and harvested for Western blot analysis using PTEN antibody. PTEN and PTEN C124S expression can be induced with Dox treatment in Clone C1 and C2, and C4 and C8, respectively. *B*, AR exists in the PTEN immunocomplex in LNCaP cells overexpressing PTEN. The stable PTEN clone (PTEN-C1) was treated with or without 4 μg/ml Dox in 10% CDS media for 24 h and treated with ethanol or 10 nM DHT for another 24 h. The cells were harvested for immunoprecipitation (IP) assay with normal mouse PTEN antibody, followed by Western blotting with AR antibody. The total cell lysates were subjected to Western blotting with PTEN and AR antibodies. *C*, Endogenous association between PTEN and AR in CWR22R cells. The IP and Western blot methods used are the same as described in (A) except that the cell lysates were from the CWR22R cells. *D*, The PTEN-AR interaction is inhibited by AR-D in CWR22R cells. The cells cultured in RPMI medium with 10% fatal calf serum were transfected with vector or FLAG-

tagged AR-D for 48 h. Cells were then harvested for immunoprecipitation (IP) assay with normal mouse IgG or PTEN antibody, followed by Western blotting with AR or FLAG antibodies. The total cell lysates were subjected to Western blotting with FLAG, PTEN, and AR antibodies.

It has been suggested that PTEN regulates the stability of $p27^{kip1}$ via a ubiquitin-proteasome pathway (3). While MG132, a proteasome inhibitor, blocked estrogen-mediated ER degradation it did not prevent PTEN-mediated AR degradation (data not shown), suggesting that PTEN promotes AR protein degradation via a proteasome-independent pathway. Interestingly, we found that the caspase-3 inhibitor DEVD-CHO, can block PTEN-mediated AR degradation (Figure 7A). We demonstrated that caspase-3 could cleave AR into three evident fragments, and DEVD-CHO completely blocked caspase-3-mediated AR degradation (Figure 7B), consistent with the previous reports (4).

As the caspase-3 inhibitor completely blocked the effect of PTEN on AR degradation, caspase-3 may mediate the PTEN-induced AR degradation. This hypothesis is further supported by our result (Figure 7C) and the earlier report showing that caspase-3 could degrade AR *in vitro* (4). It has been reported that PTEN-induced apoptosis can be rescued by caspase-3 inhibitor in LNCaP cells (5), which also strengthen our hypothesis that PTEN signaling can be mediated through caspase-3 via direct cleavage of AR protein (4). Although our study demonstrates that PTEN-mediated AR degradation is through caspase-3 activity (Figure 7), we found that the repression of AR activity by PTEN could not be rescued by a caspase-3 inhibitor or a general caspase inhibitor (Figure 7C). These contrasting results imply that PTEN suppressing AR might go through multiple pathways, and caspase-3-mediated degradation could be one of these pathways. In addition, because it has been known that caspase-3 cleaves AR at the D151 residue, we further tested the effect of PTEN on AR-D151N mutant. We found that PTEN still can repress the transactivation of AR-D151N (Figure 7C), indicating that PTEN suppressed AR not only via protein degradation. Together, our data shows it is possible that in addition to degradation of AR, direct association between AR and PTEN may also contribute to suppression of AR activity.

Figure 7



Legend Figure 7. Caspase 3 Mediates PTEN-mediated AR Degradation but not AR Transactivation

A, *In vitro* translated [S^{35}]-AR was incubated with 10 nM recombinant active caspase-3 in the presence or absence of 10 μM DEVD-CHO for 2 h, and the reaction was stopped for SDS-PAGE. *Arrowhead* indicates degraded AR fragments. B, Caspase 3 is not involved in PTEN-mediated repression of AR activity in LNCaP cells. Different passages of LNCaP cells were transfected with plasmids, as indicated, for 24 h, treated with 10 nM DHT in the presence or absence of 10 μM DEVD-CHO and 10 μM Z-VAD-FMK (a pan-caspase inbibitor) for another 24 h, and harvested for luciferase assay. C, The 293T cells were transfected with pCDNA3-FLAG-AR, pCDNA3-FLAG-AR-D151N and pCDNA3-FLAG-PTEN, as

indicated, in 10% FCS media for 16 h. The cells were harvested and assayed for luciferase activity using MMTV-luc as a reporter. AR-D151N was generated using QuikChange kit (Stratagene) with a pair of primers: 5'-GCACCTCCGGAC-GAGGATAACTCAGCTGCCCCATCCACGTTG-3' and 5'-CAACGTGGATGGGGCAGCTGAGTTATCCTCGTCCG-GAGGTGC-3'. The results were normalized by pRL-SV40 activity and the data are represented as means ± s.d of three independent experiments. (*, $p<0.05$; **, $p<0.001$ vs. control (indicated as •), Student's two-tailed *t*-test)

Key Research Accomplishments:

From 1st report

a. AR and PTEN are endogenously expressed in MCF7 cells. And we found that these two proteins can interact with each other in this cell line by co-immunoprecipitation.
b. PTEN and AR are associated directly via the PTP domain of PTEN and AR DNA binding domain.
c. AR and PTEN are endogenously expressed in MCF7 cells. And we found that these two proteins can interact with each other in this cell line by co-immunoprecipitation.
d. Using GST pull-down assay, we found that PTEN and AR are associated directly via the PTP domain of PTEN and AR DNA binding domain.
e. PTEN disrupts AR nuclear translocation and retains AR in the cytoplasm where PTEN is located.
f. PTEN suppresses AR expression resulting from reducing the AR stability by PTEN.
g. Taken together, PTEN can directly interact with AR in MCF7 and suppresses AR via interruption of nuclear translocation and protein stability.

From 2nd report

- We found that PTEN could reduce AR protein levels in COS-1 cells.
- PTEN siRNA (the most efficient one) can efficiently suppress PTEN expression and increase the AR amount and transactivation, indicating that endogenous PTEN normally inhibits AR expression and transcriptional activity
- We have demonstrated that overexpression of PTEN promotes AR degradation and suppresses AR activity.
- Transient transfection of PTEN siRNA reduced endogenous PTEN protein levels up to 50-60%, which correlated with the transfection efficiency (around 50%), reduction of PTEN expression enhanced AR protein expression in the presence and absence of androgen, and PTEN siRNA dose-dependently enhanced AR transactional activity, suggesting that endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity.
- PTEN can co-localize with AR and then influence AR nuclear translocation we assessed the immunocytofluorescence staining in COS-1 cells. AR-D, the interaction inhibitor, can block the PTEN effect on AR.
- In pulse-chase assays, PTEN clearly reduced the half-life of newly synthesized [35S]-AR 4 to 5-fold and accelerated AR degradation.

From final year:

1. Confirmed the physiological interaction between AR and PTEN by co-immunoprecipitation, by establisheing PTEN-stable LNCaP cells, using the Dox-inducible system. Dox treatment induced expression of PTEN or PTEN C124S in several clones (PTEN-C1, PTEN-C2, PTEN C124S-C4, and PTEN C124S-C8. These clones can now be used in our eventual work with breast cell lines.
2. We applied the CWR22R cell line, which endogenously expresses both AR and PTEN (Figure 6C), for co-immunoprecipitation with PTEN antibody. The results showed that AR could be detected in the PTEN-immunoprecipitated complex.
3. AR-D could also prevent endogenous PTEN from binding to AR in CWR22R cells These results suggest that AR can physiologically interact with PTEN through the AR-D region in prostate cancer cells.

4. PTEN suppressing AR might go through multiple pathways, and caspase-3-mediated degradation could be one of these pathways.. We found that PTEN still can repress the transactivation of AR-D151N, indicating that PTEN suppressed AR not only via protein degradation. Together, our data shows it is possible that in addition to degradation of AR, direct association between AR and PTEN may also contribute to suppression of AR activity.

Reportable Outcomes: A Manuscript reporting the results is in progress.

Conclusions: Our research to date has determined much about the PTEN effects on the AR, including reducing AR protein levels, inhibiting AR expression and transcriptional activity, the overexpression of PTEN promotes AR degradation and suppresses AR activity, endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity, and that PTEN can co-localize with AR and then influence AR nuclear translocation in several cell lines including some study in MCF-7 breast cancer cell lines. We also develoed clones of PTEN which can be used for future studies. We recommend the slight change in scope of our work by the addition of breast cancer cell lines other than the MCF-7. If we can adapt these studies better to the MCF-7 cells or other breast cancer cell lines we may uncover molecular mechanisms of the suppression of AR by PTEN, which in turn may imply tumor growth suppression and lead to better therapeutic strategies, for instance gene therapy, for breast cancer patients in the future.

List of people paid through this grant.

Chun-Te Wu (Ph.D) and Hiroshi Miyamoto (Ph.D.) were paid during all 3 years. Chih-Rong Shyr (graduate student) , Zhihong Dong (graduate student), Xin-Chang Zhou (graduate student), Yue Yang (Ph.D.), and Jiann-Jyh Lai (Ph.D.) were paid from 3 to 10 months during the 3 year's period.

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Appendices: None